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REGRESSION ANALYSIS FOR PREDICTING THE DURATION DEPENDENT RESPONSE OF OXIDATIVE STRESS DYNAMICS AND NUCLEAR ANOMALIES IN *CATLA CATLA* EXPOSED TO CHLORPYRIFOS AND ENDOSULFAN

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ABSTRACT

In present work, a statistical model called linear regression was applied to find out the casual relation between the dependent variables oxidative stress dynamics viz. Superoxide dismutase (SOD), peroxidase (POx), catalase (CAT) and glutathion S-transferase (GST) and nuclear abnormalities (NAs) in *Catla catla* and one independent variable exposure durations of endosulfan and chlorpyrifos (insecticides). Fish were kept under acute toxicity of insecticides mixture for 1-, 2-, 3- and 4-days. The increased SOD activity in organs of fish showed the significant and positive dependence with duration of exposure. CAT activity in gills, liver and kidney showed significantly positive relation while brain, heart and muscle tissues had significantly negative dependence with duration which showed that it decreased with increasing exposure time. The activity of POx in gills, brain and heart showed a significant dependence on exposure duration while liver, kidney and muscle had non-significant dependence with exposure time. The GST activity of fish had highly significant positive relation with exposure time. The SOD, POx and GST activities augmented in organs of fish due to mixture exposure. However, CAT activity induced in kidney, liver and gills of fish while it was decreased in heart, brain and muscles tissues. The results of geno-toxicity showed that MN (micro-nuclei) and NN had significantly positive relation with duration while other NAs had non-significant relation with time exposure. Insecticides mixture exposure increased the formation of MN and NAs in RBCs of experimental group in comparison to control group.

Keywords: Fish, Antioxidant enzymes, genotoxicity, tissues, insecticides

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INTRODUCTION

The application of pesticides in forestry, agriculture, veterinary and public health practices are gaining attention because of their potential to control pests such as weeds, insects and unwanted aquatic fauna and flora (Abu-Darwish *et al.*, 2011). The exponential use of pesticides resulted in a serious contamination of brackish and freshwater bodies which are the ultimate sink of these chemicals and their residues (Lal, 2012; Deka and Mahanta, 2016; Nugegoda and Kibria, 2017). The synthetic compounds can cause the changes in behavior and physiology of aquatic animals especially fish due to their persistent and bio-accumulation properties (Liess *et al.,* 2005). Even at very low quantity, pesticides can affect the basal metabolism. Sometimes these effects expressed as sudden mortality or by other changes like reduced growth, development and reproduction, depending on the pesticide's formulation, dose, degradation capability and also on ability of fish to metabolize them (Lal *et al.,* 2013; Sabra and Mehana, 2015). According to Chakraborty and Roy (2017) pesticides also cause alterations at biochemical and genomic level by targeting the metabolic proteins and nucleic acids, respectively.

Most commonly used pesticides in the fields includes organochlorine, organophosphate, pyrethroids, carbamates, necotenoides and trizole (Srivastava and Singh, 2014; Sarba and Mehana, 2015). Organochlorine insecticide such as endosulfan is widely used in horticultural and agricultural crops (Tuduri *et al.,* 2006; Sutherland *et al*., 2004) to minimize the insect pest's population (Rehman *et al*., 2016). Endosulfan has direct effect on fish's central nervous system resulted in hyperactivity, convulsions and sometimes cause sudden mortality. Among organophosphorus pesticides, chlorpyrifos is commonly used in agriculture and is highly toxic to fish by inhibiting the acetylcholinesterase activity in nerve cells (Yen *et al.,* 2011) and also induced gut microbiota dysbiosis, oxidative stress, neurotoxicity, disruption of endocrine and immune system (Zhang *et al*., 2017; Wang *et al*., 2019).

Pesticides are well known inducer of oxidative stress by forming the reactive oxygen species (ROS) leading to blockage of antioxidant enzymes activities

which are scavenger of free radicals. The oxidative stress in the cell can be minimized by antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) (Agarwal *et al*., 2010) and glutathion S-transferase (GST) one of the phase II enzymes protect against free radicals by conjugation (Rahaman *et al*., 1999).

Currently, micronuclei (MN) assay and other nuclear abnormalities (NAs) of erythrocytes are extensively used as bio-marker of genotoxicity and mutagen in fish exposed to contaminants (Bolognesi and Hayashi, 2011; Obiakor *et al*., 2012). This assay also identifies the single cell for NAs includes lobed, blebbed, notched and bi-nucleated cells but exact pathway of their formation is not understood (Barsiene *et al*., 2006; Da-Rocha *et al*., 2011). Ample of research was conducted on single effect of pesticides but combined impact of these chemicals is scarce. Due to above mentioned toxicity of pesticides and their increased use, this research was planned to assess the toxic effect of pesticides mixture on oxidative stress dynamics and nuclear anomalies of *Catla catla*.

MATERIALS AND METHODS

The freshwater fish, *Catla catla* were acquired from a Fish Seed Hatchery Faisalabad and shifted to wet laboratory of fisheries research Farm UAF to acclimatized the laboratory conditions for approximately a couple of weeks. For experimental trail, acclimatized fish (n=20) were placed into glass aquaria of 100 L water capacity. The physical (pH=7.25; temperature= 28°C) and chemical properties (dissolve oxygen= 5.00ppm; water hardness= 225 mgL^{-1}) of water were maintained during the experimental period. A stock-I solutions of chlorpyrifos(C) and endosulfan (E) were prepared separately by dissolving 1 g in 100ml methanol (95%). The final stock-II solutions (10 ppm) were made in deionized water while required C+E mixture (1:1) was made for treating the fish. Fish were kept under acute exposure of C+E mixture for 4-days and sampling was done at 1-day interval. The tolerance limits (LC_{50}) of *C. catla* against C+E was computed as 1.35±0.01μgL^{-1} (Naz *et al.*, 2019b).

Oxidative stress dynamics: A number (n=5) of fish were sacrificed at each sampling and organs viz. gills, liver, kidney, brain, heart and muscle were separated to assess the superoxide dismutase (SOD) by Giannopolitis and Ries (1977), peroxidase (POx) and catalase (CAT) by Chance and Mehaly (1977), and glutathione S-transferase (GST) by Mannervik (1985). Organ homogenates were prepared according to the method explained by Naz *et al*. (2019a).

Micronuclei test: For each sampling, fish blood was taken from caudal vein and smeared were made on slide and also fixed immediately with methanol for 10 minutes and stained with 10% Giemsa solution for 8 minutes (Barsiene *et al*., 2004). Micronuclei (MN) and nuclear abnormalities (NAs) including bi-nucleated cells, dumbbell shaped nuclei, blebbed, notched and de-shaped nuclei were scored according to the criterion mentioned by Fenech *et al*. (2003). The MN frequency was calculated by applying following formula:

$$MN\% = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} x100$$

Statistical Analyses: The statistically significant ($p \leq 0.05$) difference between stressed and unstressed fish antioxidant enzymes were calculated by applying ANOVA followed by Tukey test. For MN and NAs non-parametric criteria, Mann-Whitney U test was used. Relationship among various parameters and duration of exposure were found out by applying linear regression analysis. The analyses were performed in statistix 8.1 version and graphs were drawn in MS Excel.

RESULTS AND DISCUSSION

Oxidative stress dynamics: In the present research, statistical modeling called linear regression analysis was applied to find out the casual relation a dependent variable (enzymes/DNA damage) and one independent variable (duration of exposure). Table 1 shows the relationship between enzymes activities with exposure time of C+E mixture. The SOD activity in organs of fish showed the significant and positive dependence with duration of exposure. The CAT activity in gills, liver and kidney showed significantly positive relation while brain, heart and muscle had significantly negative dependence with duration which showed that it decreased with increasing exposure time. The activity of POx in gills, brain and heart showed a significant dependence on exposure duration while liver, kidney and muscle had non-significant dependence with exposure. The GST activity had highly significant positive relation with exposure time. Figure1 shows that SOD, POx and GST activities augmented in all organs of fish due to mixture exposure. However, CAT activity induced in liver, kidney and gills of fish while it was declined in heart, brain and muscles of fish. Same were reported by Unal *et al.* (2019) who noted the decrease CAT activity in brain and intestines in rotenone exposed zebra fish. Naz *et al.* (2019a) also observed augmentation in CAT activity in liver, kidney and gills of *L. rohita* after C+E exposure while it was lowered in muscle, brain and heart. Time-dependent increase in gill, kidney and liver CAT level of *L. rohita* under lethal and sub-lethal exposure of malathion was studied by Patil and David (2013). Lihocin exposure caused gradual increase in GPx and SOD of *C. catla* in gills, liver, kidney and muscle tissues exposed for 3, 7, 14, 30 and 45 days where as CAT activity is

gradually decreased throughout the study period (Vineela and Reddy, 2014). Exposure to endosulfan+deltamethrin increased the GST level in muscle, liver, kidney and gills of *Channa striata* (Abdullah *et al*., 2018). Naz *et al*. (2017) noted the time reliant increased in liver SOD level in three Indian major carps exposed to tertiary insecticides mixture of bifenthrin, chlorpyrifos and endosulfan. Naz *et al*. (2019a) also studied the increased SOD, POx and GST activities in bronchial, muscle, hepatic, neural, and cardiac tissues of *L. rohita* due to C+E exposure. Endosulfan exposure induce time-dependent raise in CAT, POD, SOD and GST activities in brain, gills, muscle and liver of *L. rohita* (Ullah *et al*., 2016). Ullah *et al*. (2014) observed the raise in CAT and POx levels in liver, brain, gills and muscle tissues of Masherr exposed to cypermethrine as exposure time increased. Activity of GST and CAT shows a significant increase afterday 1 than decreased upto 5 day in liver and brain of fish exposed to Quinalphos as compared to the control (Greeshma *et al*., 2019). Exposures of endosulfan and imidacloprid mixture on zebrafish caused time-dependent decrease in gill, liver and muscle SOD and CAT activities (Muazzam *et al*., 2019). Delthametrin triggers the CAT, GPx and SOD activities in zebra fish under acute exposure (Strungaru *et al.,* 2019). Vijayakumar *et al.* (2016) studied the accelerated SOD and CAT level in cypermethrin exposed *L. rohita*. A time-reliant significant increase in liver CAT, SOD, POx, and GST levels in rohu due to malathion exposure noted by Ullah *et al.* (2018). Pandey *et al.* (2001) documented the increased GST and GPx level of fish exposed to endosulfan while CAT activity was decreased significantly in liver, kidney and gills. Thenmozhi *et al.* (2011) assessed the higher level of gills, muscle and liver GST and CAT due to malathion in *L. rohita*. Acute (96 h) exposure of methyl parathion caused significant raised in liver, muscle, and gills SOD, CAT and GST activities of fish (Monteiro *et al*., 2006).

Nuclear Anomalies: The results of geno-toxicity showed that MN and NN had significantly positive relation with duration while other NAs had non-significant relation with time exposure (Table 2). Figure 2 showed that C+E mixture exposure increased the formation of MN and NAs in RBCs of fish in relation to control. Moreover, the high value of R^2 for fish predicts significantly high reliability of this regression model. Similarly, Ansoar-Rodriguez *et al*. (2015) documented the production of MN and NAs in blood of *Oreochromis niloticus* due to imidacloprid concentration. *Labeo rohita* showed higher frequency of MN and NAs (notched, blebbed, bi-nucleated and lobed nuclei) in blood when exposed to triazophos for 2-, 3- and 4-day of post-treatment (Ghaffar *et al.,* 2015). Significant induction of MN and NAs in *Channa punctata* erythrocytes due to deltamethrin was observed by Ansari *et al.* (2009). Anbumani and Mohankumar (2015) observed the duration-dependent induction of MN and NAs in *Catla catla* exposed to butachlor and monocrotophos mixture. Muranli and Guner (2011) noted the MN and NAs formation in erythrocytes of mosquito fish treated with lambda-cyhalothrin for 6-,12-, 24-, and 48-h. Islam *et al.* (2019) reported the increased frequency of MN in RBCs of sumithion exposed striped catfish. Wu and Ding (2016) noted the formation of MN in erythrocytes of top mouth gudgeon (*Pseudorasbora parva*) by endosulfan exposure for 48- and 96-h. Dichlorvos exposure increased the rate of MN formation in *Misgurnus anguillicaudatus* (Nan *et al*., 2015). After exposure to dimethoate, the induction of MN was increased in *Channa punctatus* as the concentration and exposure time increased (Ali *et al*., 2014). *C. chanos* showed significant increase in MN when exposed to chlorpyrifos and carbendazim (Palanikumar *et al*., 2014). *Oreochromis mossambicus* showed the high frequency of MN in erythrocytes after exposure to different pesticides viz. chlorpyrifos, cypermethrin, buctril, malathion and lambda-cyhalothrin (Naqvi *et al.,* 2016). Nwani *et al*. (2010) documented time specific MN generation in blood of carbosulfan treated *Channa punctatus* for 94-h.

Table 1: Relationships between antioxidant enzymes activities and duration of C+E mixture exposure.

Enzymes	Organs	Regression Equation	SE	r	R^2
SOD	Gills	46.4 + 0.665**Time	0.01925	0.998	0.997
	Liver	80.6 + 0.590 **Time	0.01732	0.998	0.997
	Kidney	50.5 + 0.746**Time	0.05142	0.991	0.986
	Brain	70.5 + 0.533 **Time	0.02938	0.994	0.991
	Heart	23.8 + 0.723 *Time	0.04894	0.991	0.986
	Muscle	22.0 + 0.378 *Time	0.02163	0.993	0.990
CAT	Gills	210 + 0.478 **Time	0.08546	0.940	0.910
	Liver	249 + 0.510 **Time	0.04522	0.985	0.977
	Kidney	162 + 0.412 ** Time	0.01288	0.998	0.997
	Brain	115 - 0.459** Time	0.02852	0.992	0.988
	Heart	97.4 - 0.506 ** Time	0.06152	0.971	0.957

POx	Muscle	178 - 0.486 ** Time	0.05321	0.977	0.965
	Gills	1.97 + 0.0371** Time	0.0006038	0.999	0.999
	Liver	2.74 + 0.0470 Ns Time	0.01237	0.879	0.818
	Kidney	2.11 + 0.0275 NS Time	0.007607	0.867	0.800
	Brain	2.32 + 0.0406** Time	0.001622	0.997	0.995
	Heart	1.14 + 0.0365** Time	0.0003997	0.999	0.999
	Muscle	1.13 + 0.0269NS Time	0.008510	0.833	0.749
GST	Gills	203 + 1.94**Time	0.05402	0.998	0.998
	Liver	235 + 2.46**Time	0.2285	0.983	0.975
	Kidney	232 + 2.06 **Time	0.1645	0.987	0.981
	Brain	331 + 2.26**Time	0.1635	0.990	0.985
	Heart	156 + 1.75**Time	0.2037	0.974	0.961
	Muscle	281 + 2.56**Time	0.03139	0.990	0.990

SE: Standard Error; r: Multiple Regression Coefficient; R2: Coefficient of Determination; ** Highly Significant at $p \leq 0.01$.

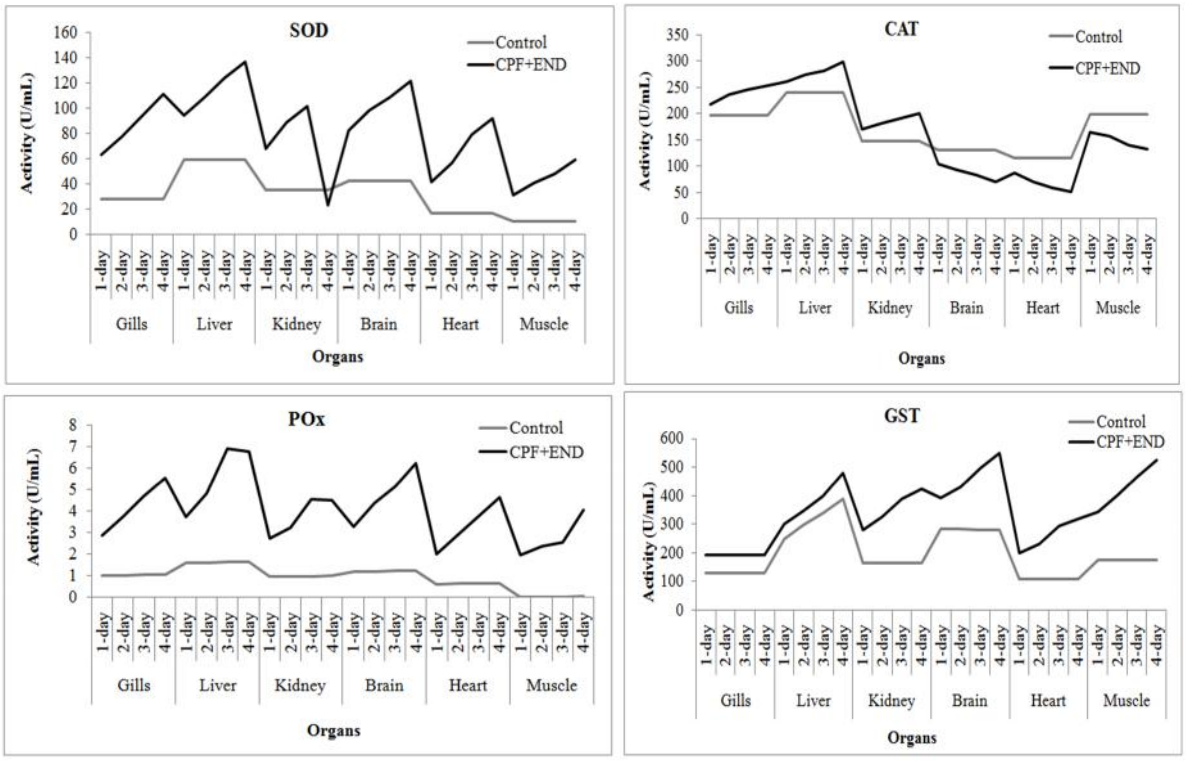

Figure 1: Activities of antioxidant enzymes in organs of fish under C+E mixture exposure at various time intervals

Table 2: Relationships between nuclear anomalies in fish and duration of C+E mixture exposure.

Nuclear Abnormalities	Regression Equation	SE	r	R^2
MN	1.05 + 0.0282 *time	0.003691	0.967	0.95
BN	0.570 + 0.0299Nstime	0.003251	0.977	0.965
DN	- 0.0200 +0.00983Ns time	0.0008457	0.985	0.978
BEN	0.105 + 0.0153Nstime	0.001808	0.973	0.959
NN	0.205 + 0.0115*time	0.0006495	0.994	0.991
DES	0.280 + 0.0495 Nstime	0.002919	0.993	0.990

SE: Standard Error; r: Multiple Regression Coefficient; R2: Coefficient of Determination; ** Highly Significant at $p \leq 0.01$.

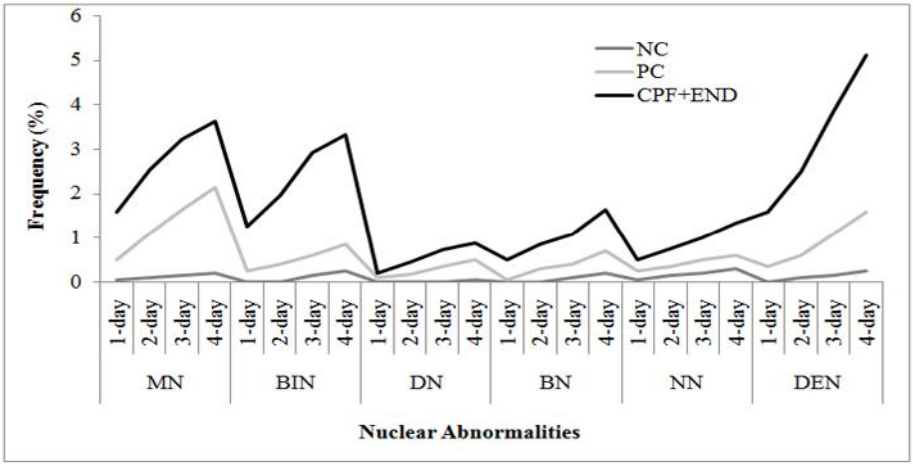

Figure 2 Nuclear anomalies in RBCs of fish under C+E mixture exposure at various time intervals

Conclusion: This study indicates a positive dependence of antioxidant enzymes activities of *Catla catla* on exposure duration of insecticides mixture. Moreover, this study also suggested that induction of MN and NAs can be used as a non-specific biomarker and can also successfully apply to evaluate geno-toxic potential of agrochemical pollutants in aquatic animals like fish.

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